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SUPPRESSION OF VASCULAR DISORDERS BY MUCOSAL ADMINISTRATION OF HEAT SHOCK PROTEIN PEPTIDES

Related Applications

This application claims priority to U.S. Provisional Patent Application No. 60/189,855, filed March 15, 2000, the entire contents of which are hereby incorporated by reference.

Field of the Invention

This invention pertains to an improvement in the treatment of vascular disorders and in the treatment of atherosclerosis in particular. More specifically, the invention is directed to the mucosal administration of heat shock protein peptides and biologically active fragments or analogs of such heat shock protein peptides for the prevention and therapeutic treatment of vascular disorders. The invention also includes aerosol, oral, and enteral formulations of heat shock protein peptides useful in the treatment of vascular disorders in mammals.

Background of the Invention

Atherosclerosis, a multifactorial process resulting in thickening of the intimal layer of arterial vessels, is characterized by an accumulation of lipids within the vessel wall and accompanying mononuclear cell infiltration and smooth muscle cell proliferation. It is a leading cause of mortality and morbidity due to cardiovascular and cerebrovascular disease in the western world. The initial lesion of atherosclerosis is the fatty streak, the focal collection of lipoprotein particles within the intima.

The current treatments for the prevention and treatment of atherosclerosis include certain pharmacological approaches, in addition to alteration of lifestyle factors which can ameliorate atherosclerosis, such as diet control, weight loss, increased exercise, and smoking cessation. Examples of pharmacological agents in current use for the treatment and prevention of atherosclerosis are hydroxymethylglutaryl-coenzyme A (HMGCoA) reductase inhibitors (statins) to control high LDL, nicotinic acid to control high lipoprotein (a) and low high density lipoprotein (HDL), and fibric acid derivatives to control high levels of triglycerides. Adjunctive pharmacological treatment includes measures directed toward control of diabetes mellitus and hypertension.

In view of the foregoing, a need still exists to develop methods and compositions for treating and/or preventing vascular disorders such as atherosclerosis. Preferably, such

methods and compositions would include non-invasive modes of administration and, more preferably, be based, in part, on the molecular interactions which mediate an inflammatory response.

Summary of the Invention

The invention solves these and other problems by providing methods and compositions for treating vascular disorders, including atherosclerosis.

It has been unexpectedly discovered by the present inventors that mucosal administration of a heat shock protein peptide, for example mycobacterial HSP65, is an effective treatment for vascular disorders, such as atherosclerosis. In one aspect, the invention is directed to a method for treating vascular disorders in a mammal in need of such treatment, comprising orally (or more generally, mucosally) administering to the mammal an effective amount of an agent comprising a heat-shock protein ("HSP"), and/or therapeutically effective fragments or analogs of a heat shock protein. Preferably, the administration is continued for a period of time sufficient to achieve at least one of the following:

(i) reduction in the level of proinflammatory Th1 cytokines;

(ii) increase in the level of anti-inflammatory Th2 cytokines; or

(iii) amelioration, retardation or suppression of at least one clinical or histological symptom of a vascular disorder.

Although not wishing to be bound to any particular theory or mechanism, it is believed that mucosally administered HSPs according to the invention can initiate immunological responses in a subject which prevent, retard or arrest an inflammatory response associated with a vascular disorder. For ease of discussion, the invention is described in terms of administering a heat shock protein; however, it is to be understood that therapeutically effective fragments or analogs of HSPs can be used in addition to or in place of HSPs to practice the claimed invention.

The present invention also relates to formulations adapted for mucosal administration, and/or delivery systems adapted from mucosal administration, comprising a HSP and useful in the treatment of vascular disorders.

It has now been discovered that an improved and more effective method for preventing or treating vascular disorders in mammals comprises mucosal administration of one or more heat shock protein peptides. Heat shock proteins (HSPs) are well known in the art and are discussed in detail below. Therapeutically effective fragments and analogs of

HSPs can be identified in screening assays which measure, e.g., any one or more of the above-listed parameters. Exemplary animal models for selecting therapeutically effective HSPs, fragments and analogs thereof, are provided in the Examples.

Thus, according to one aspect of the invention, a method for treating (including preventing) a vascular disorder in a mammal is provided. The method involves administering to a mucosal surface of the mammal at least one agent selected from the group consisting of a heat shock protein, a therapeutically effective fragment of a heat shock protein, and a therapeutically effective analog of a heat shock protein, wherein the agent is present in an effective amount for treating the disorder. In certain embodiments, the vascular disorder is an inflammatory vascular disorder and the effective amount is an amount sufficient to suppress, in whole or in part, the inflammatory response.

According to one embodiment of this aspect of the invention, the mucosal surface includes nasal epithelium. In a second embodiment the mucosal surface includes oral mucosa. In yet other embodiments the mucosal surface includes a luminal surface of a gastrointestinal organ selected from the group consisting of: stomach, small intestine, large intestine, and rectum. In certain embodiments of this aspect of the invention, the disorder includes a cell-mediated immune response. In certain other embodiments, the disorder includes an antibody-mediated immune response. In a preferred embodiment of this aspect of the invention, the disorder is atherosclerosis. In another preferred embodiment of this aspect of the invention, the heat shock protein is mycobacterial HSP65. In another preferred embodiment of this aspect of the invention, the heat shock protein is human HSP60. In yet another preferred embodiment of this aspect of the invention, the heat shock protein is chlamydial HSP60.

In a second aspect, the present invention provides a method for treating a vascular disorder (e.g., an inflammatory vascular disorder) in a mammal. The method includes administering to the mammal by inhalation an effective amount of a composition containing at least one agent selected from the group consisting of a heat shock protein, a therapeutically effective fragment of a heat shock protein, and a therapeutically effective analog of a heat shock protein, wherein the agent is present in an effective amount for treating the disorder. In certain embodiments, the vascular disorder is an inflammatory vascular disorder and the effective amount is an amount sufficient to suppress, in whole or in part, the inflammatory response.

In certain embodiments of this aspect of the invention, the disorder includes a cell-mediated immune response. In certain other embodiments, the disorder includes an antibody-mediated immune response. In a preferred embodiment of this aspect of the invention, the disorder is atherosclerosis. In another preferred embodiment of this aspect of the invention, the heat shock protein is mycobacterial HSP65. In another preferred embodiment of this aspect of the invention, the heat shock protein is human HSP60. In yet another preferred embodiment of this aspect of the invention, the heat shock protein is chlamydial HSP60. In yet another preferred embodiment of this aspect of the invention, the agent is in aerosol form.

In a third aspect, the present invention provides a method for suppressing a vascular disorder in a mammal, which includes administering to the mammal via the pulmonary tract an effective amount of a composition comprising at least one member selected from the group consisting of a heat shock protein, a therapeutically effective fragment of a heat shock protein, and a therapeutically effective analog of a heat shock protein, wherein the agent is present in an effective amount for treating the disorder. In certain embodiments, the vascular disorder is an inflammatory vascular disorder and the effective amount is an amount sufficient to suppress, in whole or in part, the inflammatory response. In a preferred embodiment of this aspect of the invention, the disorder is atherosclerosis. In another preferred embodiment of this aspect of the invention, the heat shock protein is mycobacterial HSP65. In another preferred embodiment of this aspect of the invention, the heat shock protein is human HSP60. In yet another preferred embodiment of this aspect of the invention, the heat shock protein is chlamydial HSP60. In yet another preferred embodiment of this aspect of the invention, the agent is in aerosol form.

The invention provides in yet another aspect a method for treating a vascular disorder in a mammal, which includes orally or enterally administering to the mammal an effective amount of a composition containing at least one agent selected from the group consisting of a heat shock protein, a therapeutically effective fragment of the heat shock protein, and a therapeutically effective analog of the heat shock protein, wherein the agent is present in an effective amount for treating the disorder. In certain embodiments, the vascular disorder is an inflammatory vascular disorder and the effective amount is an amount sufficient to suppress, in whole or in part, the inflammatory response.

In certain embodiments of this aspect of the invention, the disorder includes a cell-mediated immune response. In certain other embodiments, the disorder includes an antibody-mediated immune response. In a preferred embodiment of this aspect of the invention, the

disorder is atherosclerosis. In another preferred embodiment of this aspect of the invention, the heat shock protein is mycobacterial HSP65. In another preferred embodiment of this aspect of the invention, the heat shock protein is human HSP60. In yet another preferred embodiment of this aspect of the invention, the heat shock protein is chlamydial HSP60. According to one embodiment of this aspect of the invention, the composition is administered orally. According to another embodiment of this aspect of the invention, the composition is administered enterally. In certain embodiments the composition is administered in solid form. In certain other embodiments the composition is administered in semi-solid form. In yet other embodiments the composition is administered in liquid form. In other embodiments the administered composition further includes a pharmaceutically acceptable carrier.

In yet another aspect the present invention provides a composition for treating a vascular disorder in a mammal, which includes at least one agent selected from the group consisting of a heat shock protein, a therapeutically effective fragment of the heat shock protein, and a therapeutically effective analog of the heat shock protein, wherein the agent is present in an effective amount for treating the disorder. In certain embodiments, the vascular disorder is an inflammatory vascular disorder and the effective amount is an amount sufficient to suppress, in whole or in part, the inflammatory response.

In certain embodiments of this aspect of the invention, the disorder includes a cell-mediated immune response. In certain other embodiments, the disorder includes an antibody-mediated immune response. In a preferred embodiment of this aspect of the invention, the disorder is atherosclerosis. In another preferred embodiment of this aspect of the invention, the heat shock protein is mycobacterial HSP65. In another preferred embodiment of this aspect of the invention, the heat shock protein is human HSP60. In yet another preferred embodiment of this aspect of the invention, the heat shock protein is chlamydial HSP60. In certain embodiments the composition is administered in solid form. In certain other embodiments the composition is administered in semi-solid form. In yet other embodiments the composition is administered in liquid form. In yet other embodiments the composition is administered in aerosol form. In certain embodiments of this aspect of the invention, the composition further includes a pharmaceutically acceptable carrier.

These and other aspect of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments and to the accompanying drawings.

All documents identified in this application are incorporated in their entirety herein by reference.

Brief Description of the Drawings

Figure 1 is a graph showing HSP65-induced proliferation of popliteal lymph node cells from C57BL/6 mice nasally treated with 0.8 μg HSP65 or 0.8 μg ovalbumin (OVA) peptide prior to immunization with HSP65 in complete Freund's adjuvant (CFA).

Figure 2 is a pair of graphs showing HSP65-induced secretion of (A) IFN-γ and (B) IL-10 by popliteal lymph node cells from C57BL/6 mice nasally treated with 0.8 μg HSP65 or 0.8 μg OVA peptide prior to immunization with HSP65 in CFA.

Figure 3 is a graph showing HSP65-induced proliferation of popliteal lymph node cells from C57BL/6 mice orally treated (fed) with 8 μg of HSP65 or OVA peptide prior to immunization with HSP65 in incomplete Freund's adjuvant (IFA) or CFA.

Figure 4 is a pair of graphs showing the HSP65-induced secretion of (A) IFN-γ and (B) IL-6 by popliteal lymph node cells from C57BL/6 mice orally treated (fed) with 8 μg of HSP65 or OVA peptide prior to immunization with HSP65 in IFA or CFA.

Figure 5 is a graph showing the aortic arch plaque size (μm^2) after 8 weeks on a high cholesterol diet in wild-type C57BL/6mice and in low density lipoprotein receptor knockout (LDLr -/-) C57BL/6 mice nasally treated with OVA peptide (OVA), nasally treated with HSP65 (HSP Nasal), or orally treated with HSP65 (HSP Fed). Data represent mean ± standard deviation values (n=8), and asterisks (*) denote $p<0.05$ determined by Student's T-test vs. OVA treated.

Figure 6 is a graph showing the percent aortic arch area stained with anti-Mac-3 antibodies after 8 weeks on a high cholesterol diet in wild-type C57BL/6 mice and in LDLR -/- C57BL/6 mice nasally treated with OVA peptide, nasally treated with HSP65, and orally treated with HSP65.

Figure 7 is a graph showing the percent aortic arch area stained with anti-CD4 antibodies after 8 weeks on a high cholesterol diet in wild-type C57BL/6 mice and in LDLR -/- C57BL/6 mice nasally treated with OVA peptide, nasally treated with HSP65, and orally treated with HSP65.

The figures are illustrative and are not essential to enablement of the inventions disclosed herein.

These and other embodiments of the subject invention will be described in the detailed description.

Detailed Description of the Invention

All publications and patent applications listed herein are hereby incorporated by reference.

The following terms as used in this disclosure have the meanings ascribed to them below.

"Mammal" is defined herein as any warm-blooded higher vertebrate organism (including a human) having an immune system and being susceptible to vascular disorders or an induced or spontaneous animal model thereof.

"Abatement", "suppression" or "reduction" of an immune response or reaction includes partial reduction or amelioration of one or more symptoms of the attack or reaction, e.g., reduction in number of activated T cells or in number of antibodies or in the levels of at least one proinflammatory cytokine (e.g., interferon (IFN)-γ, interleukin (IL)-2 or tumor necrosis factor (TNF)) or an increase in the levels of at least one anti-inflammatory cytokine, such as transforming growth factor (TGF)-β, IL-4, IL-10.

As employed herein the term "treatment" refers to treatment of an active disorder in an affected individual or to prophylactic administration to prevent a disorder in a susceptible individual. "Treatment" of vascular disorders such as atherosclerosis is intended to include, although not be limited to, one or more of the following:

(i) alteration of the profile of one or more cytokines in vascular disorder patient or animal model so that it conforms to or approaches the cytokine profile of a sex-and-age-matched subject without a vascular disorder;

(ii) delay in the progress of vascular disorder-related symptoms (detectable by methods known in the art);

(iii) amelioration of the vascular disorder as evaluated by one or more of the symptoms, factors and criteria as in (ii) of this definition, or by observation by a physician specializing in vascular disorders, or

(iv) decrease in the amount of vascular inflammation compared to the amount that would otherwise have been observed absent the treatment. Accordingly, treatment includes (a) delaying or preventing onset of clinical symptoms of vascular disorder, which is prevention of the clinical symptoms of disorder; (b) abating or arresting, delaying or

preventing an abnormal inflammatory response (which may or may not be accompanied by clinical symptoms) which can have the attributes of both therapy (if the occurrence of inflammation precedes the treatment) and prevention (to the extent that inflammation, if unchecked, would later cause clinical symptoms); and (c) treating vascular disorder after the appearance of clinical symptoms, which is therapy. The clinical symptoms referred to above include those recognized by physicians and others skilled in the art and may include those described in, for example, Harrison's Principles of Internal Medicine, 14th ed., AS Fauci et al., eds., New York: McGraw-Hill, 1998.

Mucosal tolerance according to the invention is an advantageous method for treating vascular disorders for several reasons:

(1) Absence of toxicity. For example, no toxicity has been observed in clinical trials or animal experiments involving oral or other mucosal administration of other protein antigens, such as bovine myelin (which contains myelin basic protein (MBP) and proteolipid protein (PLP)) to humans afflicted with multiple sclerosis, or oral or by-inhalation administration of chicken Type II collagen to humans or rodents afflicted with rheumatoid arthritis (or a corresponding animal model disorder); or oral administration of bovine S-antigen to humans afflicted with uveoretinitis; or oral administration of insulin to healthy volunteers.

(2) Containment of immunosuppression. Conventional treatments of immune system disorders involve administration of non-specific immunosuppressive agents, such as the cytotoxic drugs methotrexate, cyclophosphamide (CYTOXAN®, Bristol-Myers Squibb), azathioprine (IMURAN®, Glaxo Wellcome) and cyclosporin A (SANDIMMUNE®, NEORAL®, Novartis). Steroid compounds such as prednisone and methylprednisolone (also non-specific immunosuppressants) are also employed in many instances. All of these currently employed drugs have limited efficacy (e.g., against both cell-mediated and antibody-mediated autoimmune disorders). Furthermore, such drugs have significant toxic and other side effects and, more important, eventually induce "global" immunosuppression in the subject being treated. Prolonged treatment with the drugs down-regulates the normal protective immune response against pathogens, thereby increasing the risk of infection. In addition, patients subjected to prolonged global immunosuppression have an increased risk of developing severe medical complications from the treatment such as malignancies, kidney failure and diabetes.

(3) Convenience of therapy. Mucosal administration is more convenient than parenteral, or other forms, of administration.

The present inventors carried out a series of experiments to discover whether mucosal tolerization techniques causes suppression of inflammation characteristic of atherosclerosis. The use of the mucosal route to induce tolerance against an autoimmune response was found effective in an animal model to suppress undesirable immune response associated with atherosclerosis, and to reduce symptoms of the disorder in an animal model. The Examples include a detailed description of these experiments.

The present method of treating vascular disorders, particularly atherosclerosis, is based on the mucosal administration results, in one embodiment, in regulation of the specific immune response thought to be associated with the disorder. While not wishing to be bound by theory, the present inventors believe that suppression of this response is effected primarily by an active suppression mechanism, i.e., the elicitation of T cells characterized by an anti-inflammatory cytokine profile.

"Mucosal" administration includes oral, enteral, intragastic, nasal, buccal or intrapulmonary administration, and more generally any method of administration (e.g., by inhalation) of an active ingredient that brings the ingredient in contact with the immune system of the treated subject at the mucosa-associated lymphoid tissue (MALT), including that of the gut, nasal, buccal, bronchial or pulmonary mucosa.

In one embodiment, the present invention provides a method for treating a mammal suffering from (or at risk for developing) vascular disorders comprising mucosally administering to the mammal an effective amount of a composition comprising a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog. Administration is preferably continued for a period of time sufficient to achieve a change in one of the parameters described above. In a preferred embodiment, the present invention provides the method as described wherein the mammal is human.

The present invention also provides a pharmaceutical formulation for administering to a mammal suffering from a vascular disorder, comprising an oral or other mucosal dosage form and delivery system containing an effective amount of a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog that is sufficient to achieve at least one of the above-described measures of treatment.

In one embodiment, the present invention provides the above-described pharmaceutical formulation wherein the oral dosage form is a solid dosage form selected

from the group consisting of a tablet, a capsule and a caplet. In another embodiment, the present invention provides the pharmaceutical formulation as described above wherein the oral dosage form comprises an aqueous suspension solution of a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog. In additional embodiments, the present invention provides the pharmaceutical formulation as above-described further comprising a pharmaceutically acceptable carrier or diluent.

The present invention also provides a pharmaceutical formulation for administering to a mammal suffering from a vascular disorder comprising a dosage form according to the invention adapted for nasal or buccal administration. In one embodiment, the present invention provides the pharmaceutical formulation in aerosol or spray form to be delivered by inhalation as described above further comprising a pharmaceutically acceptable carrier or diluent. The formulation can, for example, be administered in a nebulizer or inhaler.

Antigens that May be Used to Induce Tolerance

It has now been discovered that mucosal administration of heat shock protein peptides (or therapeutically effective fragments or analogs thereof), particularly in aerosol form, is effective in treating atherosclerosis in mammals. A particularly surprising and unexpected development is the discovery that administration of heat shock protein peptides in aerosol form is more effective in preventing and treating atherosclerosis in mammals than administration of the same heat shock protein peptides in solid form via the oral route. Also surprising is the discovery that it is possible to achieve effective suppression and prevention of vascular disorders in mammals using a smaller quantity of such heat shock protein peptides in an aerosol form than by administration of a solid dosage form. The aerosol administration of heat shock protein peptides has been found to be effective in suppressing both cell-mediated and antibody-mediated inflammatory responses associated with atherosclerosis.

The heat shock proteins are a family of approximately twenty-five highly conserved proteins upregulated in response to various forms of stress. Heat shock proteins are among the most highly conserved proteins in existence. Generally HSPs are survival factors, involved in protein folding, assembly of protein complexes, and prevention of protein unfolding. Certain heat shock proteins are also known as chaperonin proteins and as GroE or GroEL proteins. Several reviews of heat shock proteins have been published, including Lindquist S (1986) *Ann Rev Biochem* 55:1151-91; Pelham HR (1986) *Cell* 46:959-61; Lindquist S and Craig EA (1988) *Ann Rev Genet* 22:631-77; Pelham HR (1989) *EMBO J*

8:3171-6; Schlesinger MJ (1990) *J Biol Chem* 265:12111-4; Kaufmann SH (1990) *Immunol Today* 11:129-37; Morimoto RI (1991) *Cancer Cells* 3:295-301; and Nover L (1991) *Nature New Biol* 3:855-9. In recent years it has been reported that HSPs can play important roles in the development of disorders such as rheumatoid arthritis, insulin-dependent diabetes mellitus, and multiple sclerosis.

Heat shock proteins may be obtained from any suitable source, including from bacterial, mycobacterial, vertebrate, mammalian (e.g., human), invertebrate, and plant (including yeast) sources. Preferred categories of heat shock proteins include those derived from mycobacterial, mammalian (e.g., human), and bacterial sources.

As used herein the term “HSP65” refers to a particular 65 kDa isoform of heat shock protein derived from *Mycobacterium tuberculosis*. HSP65 is a 540 amino acid residue protein described in Shinnick TM *J Bacteriol* 169:1080-8 (1987) and GenBank Accession No. A26950. The amino acid sequence for HSP65 is presented as SEQ ID NO:1.

SEQ ID NO:1

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MAKTIAYDEE ARRGLERGLN ALADAVKVTL GPKGRNVVLE KKWGAPTITN DGVSIAKEIE  60
LEDPYEKIGA ELVKEVAKKT DDVAGDGTTT ATVLAQALVR EGLRNVAAGA NPLGLKRGIE 120
KAVEKVTETL LKGAKEVETK EQIAATAAIS AGDQSIGDLI AEAMDKVGNE GVITVEESNT 180
FGLQLELTEG MRFDKGYISG YFVTDPERQE AVLEDPYILL VSSKVSTVKD LLPLLEKVIG 240
AGKPLLIIAE DVEGEALSTL VVNKIRGTFK SVAVKAPGFG DRRKAMLQDM AILTGGQVIS 300
EEVGLTLENA DLSLLGKARK VVVTKDETTI VEGAGDTDAI AGRVAQIRQE IENSDSDYDR 360
EKLQERLAKL AGGVAVIKAG AATEVELKER KHRIEDAVRN AKAAVEEGIV AGGGVTLLQA 420
APTLDELKLE GDEATGANIV KVALEAPLKQ IAFNSGLEPG VVAEKVRNLP AGHGLNAQTG 480
VYEDLLAAGV ADPVKVTRSA LQNAASIAGL FLTTEAVVAD KPEKEKASVP GGGDMGGMDF 540
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The complete sequence structure for this and other heat shock protein peptides are available on the GenBank public database.

As used herein the term “HSP65” also refers to a particular 65 kDa isoform of heat shock protein derived from *Mycobacterium bovis* Bacillus Calmette-Guerin (BCG), as the protein sequence of this HSP65 identical to that of HSP65 derived from *Mycobacterium tuberculosis*. Thole JE et al. *Infect Immun* 55:1466-75 (1987); GenBank Accession No. P06806. HSP65 can further include any polypeptide having the same amino acid sequence as SEQ ID NO:1 as well as polypeptides having a substantial degree of homology to SEQ ID NO:1.

In certain preferred embodiments peptide fragments of HSP65 are peptides at least 10 amino acids long that occur between amino acid residues 201-300 of HSP65. These HSP65 fragments include, for example, fragments having amino acid residues 201-210, 211-220,

221-230, 231-240, 241-250, 251-260, 261-270, 271-280, 281-290, or 291-300. Also included as HSP65 fragments are fragments having amino acid residues 192-201, 202-211, 212-221, 222-231, 232-241, 242-251, 252-261, 262-271, 272-281, 282-291, or 292-301; fragments having amino acid residues 193-203, 203-212, 213-222, 223-232, 233-242, 243-252, 253-262, 263-272, 273-282, 283-292, or 293-302; and so on, i.e., every 10-mer containing at least one amino acid residue between 201-300.

As used herein the term "human HSP60" refers to a particular 60 kDa isoform of heat shock protein derived from humans. Human HSP60 is a 573 amino acid residue protein described in Jindal S et al. *Mol Cell Biol* 9:2279-83 (1989) and GenBank Accession No. AAA60127. The amino acid sequence of HSP60 is presented as SEQ ID NO:2. Human HSP60 can also include any polypeptide with the same amino acid sequence as SEQ ID NO:2 as well as polypeptides having a substantial degree of homology to SEQ ID NO:2.

SEQ ID NO:2

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MLRLPTVFRQ MRPVSRVLAP HLTRAYAKDV KFGADARALM LQGVDLLADA VAVTMGPKGR  60
TVIIEQSWGS PKVTKDGVTV AKSIDLKDKY KNIGAKLVQD VANNTNEEAG DGTTTATVLA 120
RSIAKEGFEK ISKGANPVEI RRGVMLAVDA VIAELKKQSK PVTTPEEIAQ VATISANGDK 180
EIGNIISDAM KKVGRKGVIT VKDGKTLNDE LEIIEGMKFD RGYISPYFIN TSKGQKCEFQ 240
DAYVLLSEKK ISSIQSIVPA LEIANAHRKP LVIIAEDVDG EALSTLVLNR LKVGLQVVAV 300
KAPGFGDNRK NQLKDMAIAT GGAVFGEEGL TLNLEDVQPH DLGKVGEVIV TKDDAMLLKG 360
KGDKAQIEKR IQEIIEQLDV TTSEYEKEKL NERLAKLSDG VAVLKVGGTS DVEVNEKKDR 420
VTDALNATRA AVEEGIVLGG GCALLRCIPA LDSLTPANED QKIGIEIIKR TLKIPAMTIA 480
KNAGVEGSLI VEKIMQSSSE VGYDAMAGDF VNMVEKGIID PTKVVRTALL DAAGVASLLT 540
TAEVVVTEIP KEEKDPGMGA MGGMGGGMGG GMF                              573
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As used herein the term "chlamydial HSP60" refers to a particular 60 kDa isoform of heat shock protein derived from various species of Chlamydia, including *Chlamydophila pneumoniae*. Chlamydial HSP60 is a 544 amino acid residue protein described in Kikuta LC et al. (1991) *Infect Immun* 59:4665-9, Kalman S et al. (1999) *Nat Genet* 21:385-9, and GenBank Accession No. AAD18287. The amino acid sequence of chlamydial HSP60 is presented as SEQ ID NO:3. Chlamydial HSP60 can also include any polypeptide with the same amino acid sequence as SEQ ID NO:3 as well as polypeptides having a substantial degree of homology to SEQ ID NO:3, e.g., HSP60 derived form other chlamydia species. Yuan M et al., (1992) Infect Immun 60:2288-96.

SEQ ID NO:3

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MAAKNIKYNE EARKKIHKGV KTLAEAVKVT LGPKGRHVVI DKSFGSPQVT KDGVTVAKEI  60
ELEDKHENMG AQMVKEVASK TADKAGDGTT TATVLAEAIY SEGLRNVTAG ANPMDLKRGI 120
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DKAVKVVVDE LKKISKPVQH HKEIAQVATI SANNDSEIGN LIAEAMEKVG KNGSITVEEA 180
KGFETVLDVV EGMNFNRGYL SSYFSTNPET QECVLEDALI LIYDKKISGI KDFLPVLQQV 240
AESGRPLLII AEEIEGEALA TLVVNRLRAG FRVCAVKAPG FGDRRKAMLE DIAILTGGQL 300
VSEELGMKLE NTTLAMLGKA KKVIVTKEDT TIVEGLGNKP DIQARCDNIK KQIEDSTSDY 360
DKEKLQERLA KLSGGVAVIR VGAATEIEMK EKKDRVDDAQ HATIAAVEEG ILPGGGTALV 420
RCIPTLEAFL PMLANEDEAI GTRIILKALT APLKQIASNA GKEGAIICQQ VLARSANEGY 480
DALRDAYTDM IDAGILDPTK VTRSALESAA SIAGLLLTTE ALIADIPEEK SSSAPAMPSA 540
GMDY                                                              544
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As used herein the terms "mycobacterial HSP65," "human HSP60," and "chlamydial HSP60" also embrace homologs and alleles of mycobacterial HSP65, human HSP60, and chlamydial HSP60, respectively. In general homologs and alleles typically will share at least 40% nucleotide identity and/or at least 50% amino acid identity to the sequences of specified nucleic acids and polypeptides, respectively. Thus homologs and alleles of mycobacterial HSP65, human HSP60, and chlamydial HSP60 typically will share at least 40% nucleotide identity and/or at least 50% amino acid identity to the sequences of mycobacterial HSP65, human HSP60, and chlamydial HSP60 nucleic acids and polypeptides, respectively. In some instances homologs and alleles will share at least 50% nucleotide identity and/or at least 65% amino acid identity and in still other instances will share at least 60% nucleotide identity and/or at least 75% amino acid identity. Preferably the homologs and alleles will share at least 80% nucleotide identity and/or at least 90% amino acid identity, and more preferably will share at least 90% nucleotide identity and/or at least 95% amino acid identity. Most preferably the homologs and alleles will share at least 95% nucleotide identity and/or at least 99% amino acid identity. The homology can be calculated using various publicly available software tools developed by the National Center for Biotechnology Information (NCBI, Bethesda, Maryland) that can be obtained through the internet (ftp:/ncbi.nlm.nih.gov/pub/). Exemplary tools include the BLAST system available from the NCBI at http://www.ncbi.nlm.nih.gov, used with default settings. Pairwise and ClustalW alignments (BLOSUM30 matrix setting) as well as Kyte-Doolittle hydropathic analysis can be obtained, for example, using the MacVector sequence analysis software (Oxford Molecular Group). Watson-Crick complements of the foregoing nucleic acids also are embraced by the invention. Nonlimiting examples of HSP homologs are provided in Table 1.

Table 1. GenBank accession numbers for exemplary homologs of SEQ ID NO's 1-3

	Accession No.	Source	Length
Mycobacterial HSP65			
	AAA25354.1	*M. leprae*	588
	AAA99670.2	*M. avium*	582
	AAB49990.1	*T. tyrosinosolvens*	539
	AAF33788.1	*P. acnes*	544
	AAF91444.1	*M. avium*	541
	BAB12250.1	*P. granulosum*	533
	CAB93056	*S. coelicolor*	541
	O33658	*S. lividans*	477
	P06806	*M. bovis*	540
	P09239	*M. leprae*	541
	P42384	*M. paratuberculosis*	541
	Q00768	*S. albus*	540
Human HSP60			
	A34173	Chinese hamster	573
	AAB94640.1	*C. variipennis*	581
	AAD27589.1	*O. volvulus*	598
	CAA10230.1	*P. acuminatus*	580
	CAA37653.1	mouse	555
	CAA37654.1	rat	547
	CAB56199.1	*P. lividus*	582
	CAB58441	*M. persicae*	573
	I53052	human	573
	O02649	*D. melanogaster*	573
	P19226	mouse	573
	P29185	*Z. mays*	577
	P50140	*C. elegans*	568
	S13089	rat	573
Chlamydial HSP60			
	A60273	*C. trachomatis*	544
	AAA19871	*C. muridarum*	544
	AAA23128	*C. trachomatis*	544
	AAA97911	*C. muridarum*	544
	AAD18287	*C. pneumoniae*	544
	AAD18915	*C. pneumoniae*	526
	AAD19036	*C. pneumoniae*	519
	AAD26143	*C. pecorum*	497
	AAD26144	*C. abortus*	497
	AAD26145	*C. pneumoniae*	497
	B41479	*C. trachomatis*	544
	BAA98985	*C. pneumoniae*	526
	BAA99106	*C. pneumoniae*	519
	CAA42673	*C. pneumoniae*	544
	D72036	*C. pneumoniae*	526

I40731	*C. trachomatis*	544
P15599	*C. psittaci*	544
P17203	*C. trachomatis*	544
P31681	*C. pneumoniae*	544
Q59322	*C. muridarum*	544
S19023	*C. pneumoniae*	544

The invention also embraces variants of the preferred HSP protein peptides described above. As used herein, a “variant” of a protein peptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of a protein peptide. Accordingly, a “variant” of an HSP protein peptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of an HSP protein peptide. Modifications which create an HSP variant can be made to an HSP protein peptide for a variety of reasons, including 1) to reduce or eliminate an activity of an HSP protein peptide; 2) to enhance a property of an HSP protein peptide; 3) to provide a novel activity or property to an HSP protein peptide; or 4) to establish that an amino acid substitution does or does not affect HSP protein peptide activity. Modifications to an HSP protein peptide are typically made to the nucleic acid which encodes the HSP protein peptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the protein peptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety (for example, biotin, fluorophore, radioisotope, enzyme, or peptide), addition of a fatty acid, and the like.

Modifications also embrace fusion proteins comprising all or part of the HSP amino acid sequence. One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus “design” a variant HSP according to known methods. One example of such a method is described by Dahiyat and Mayo in *Science* 278:82-87 (1997), whereby proteins can be designed *de novo*. The method can be applied to a known protein to vary a only a portion of the polypeptide sequence. By applying the computational methods of Dahiyat and Mayo, specific variants of an HSP protein peptide can be proposed and tested to determine whether the variant retains a desired conformation.

Variants include HSP protein peptides which are modified specifically to alter a feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of an HSP protein peptide by

eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encode an HSP protein peptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the variant polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with a desired property. Further mutations can be made to variants (or to non-variant HSP protein peptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in a particular host. The preferred codons for translation of a nucleic acid in, e.g., *E. coli*, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of an HSP gene or cDNA clone to enhance expression of the polypeptide.

The activity of variants of HSP protein peptides can be tested by cloning the gene encoding the variant HSP protein peptide into a prokaryotic or eukaryotic (e.g., mammalian) expression vector, introducing the vector into an appropriate host cell, expressing the variant HSP protein peptide, and testing for a functional capability of the HSP protein peptides as disclosed herein. For example, the variant HSP protein peptide can be tested for its ability to suppress a vascular disorder, as set forth below in the examples.

The skilled artisan will also realize that conservative amino acid substitutions may be made in HSP protein peptides to provide functionally equivalent variants of the foregoing polypeptides, i.e., variants which retain the functional capabilities of the HSP protein peptides. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, FM Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent variants of the

HSP protein peptides include polypeptides having conservative amino acid substitutions of SEQ ID NOs:1-3. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

HSPs useful in the practice of the invention can be obtained through any method known in the art. HSP65, human HSP60, and chlamydial HSP60 can be obtained from a variety of commercial sources, prepared from natural sources using conventional protein preparatory methods, or expressed as recombinant proteins. Purified HSP65 can be obtained from commercial sources such as StressGen Biotechnologies Corporation, Vancouver, BC, Canada. Purified recombinant human HSP60 can be obtained from commercial sources such as StressGen Biotechnologies Corporation, Vancouver, BC, Canada.

In preferred embodiments the HSP is isolated. As used herein with reference to an HSP, the term "isolated" means removed from its natural biological surroundings, i.e., from the cells in which they occur in nature. An isolated HSP can but need not necessarily be purified. In preferred embodiments the HSP peptide fragments and HSP peptide analogs are isolated. As used herein with reference to HSP peptide fragments and HSP peptide analogs, the term "isolated" means removed from its natural biological surroundings or biological materials. For example, if the HSP peptide fragments or HSP analogs are prepared by recombinant cloning techniques, the HSP peptide fragments or HSP analogs are separated from the cells used to express them. Isolated HSP peptide fragments or HSP analogs can but need not necessarily be purified.

HSP fragments useful according to the invention are preferably immunogenic and can be derived from the HSPs through any method known in the art. For example, the fragments can be prepared as synthetic oligopeptides as described further herein. The HSP peptide fragments will typically be at least 10 amino acids long, and may be at least 15, 16, 17, 18, 19, 20 or more consecutive amino acids in length. HSP peptide fragments can include any HSP-specific peptide at least one residue shorter than the corresponding full-length HSP.

Formulations for Mucosal Administration

Administration of more than one antigen is possible, and may be desirable (e.g., when the patient's immune T cells recognize more than one antigen).

Suitable formulations according to the invention include formulations adapted for oral, enteral, buccal, nasal, bronchial or intrapulmonary administration. The preparation of such formulations is well within the skill of the art. It is preferred that such formulations not

contain substances that can act as adjuvants in order to avoid sensitization of the treated subject. It is also preferred that the antigens employed be of synthetic provenance and not isolated from biological sources to avoid the risk of infection (notably, but not exclusively, to avoid transmission of agent responsible for the Creutzfeld-Jacob disease). Additionally, it is preferred that the formulation not contain adsorption promoting agents or ingredients that protect against proteolytic degradation.

Suitable oral formulations for use in tolerization of T-cell mediated immune responses according to the present invention can be in any suitable orally administrable form, for example, a pill, a liquid, or a capsule or caplet containing an effective amount of antigen. Each oral formulation may additionally comprise inert constituents including pharmaceutically acceptable carriers, diluents, fillers, disintegrants, flavorings, stabilizers, preservatives, solubilizing or emulsifying agents and salts as is well-known in the art. For example, tablets may be formulated in accordance with conventional procedures employing solid carriers and other excipients well-known in the art. Capsules may be made from any cellulose derivatives. Nonlimiting examples of solid carriers include starch, sugar, bentonite, silica and other commonly used inert ingredients. Diluents for liquid oral formulations can include *inter alia* saline, syrup, dextrose and water.

The antigens (i.e., HSPs and therapeutically effective fragments and analogs thereof) used in the present invention can also be made up in liquid formulations or dosage forms such as, for example, suspensions or solutions in a physiologically acceptable aqueous liquid medium. Such liquid media include water, or suitable beverages, such as fruit juice or tea which will be convenient for the patient to sip at spaced apart intervals throughout the day. When given orally in liquid formulations the antigen may be dissolved or suspended in a physiologically acceptable liquid medium, and for this purpose the antigen may be solubilized by manipulation of its molecule (e.g., hydrolysis, partial hydrolysis or trypsinization) or adjustment of the pH within physiologically acceptable limits (e.g., 3.5 to 8). Alternatively, the antigen may be reduced to micronized form and suspended in a physiologically acceptable liquid medium, or in a solution.

Sustained release oral delivery systems are also contemplated and are preferred. Nonlimiting examples of sustained release oral dosage forms include those described in U.S. Patent No. 4,704,295, issued November 3, 1987; U.S. Patent No. 4,556,552, issued December 3, 1985; U.S. Patent No. 4,309,404, issued January 5, 1982; U.S. Patent No. 4,309,406, issued January 5, 1982; U.S. Patent No. 5,405,619, issued April 10, 1995; PCT International

Application WO 85/02092, published May 23, 1985; U.S. Patent No. 5,416,071, issued May 16, 1995; U.S. Patent No. 5,371,109, issued December 6, 1994; U.S. Patent No. 5,356,635, issued Oct. 18, 1994; U.S. Patent No. 5,236,704, issued August 17, 1993; U.S. Patent No. 5,151,272, issued September 29, 1992; U.S. Patent No. 4,985,253, issued January 15, 1991; U.S. Patent No. 4,895,724, issued January 23, 1990; and U.S. Patent No. 4,675,189, issued June 23, 1987.

Sustained release oral dosage forms coated with bioadhesives can also be used. Examples are compositions disclosed in European Published Application EP 516141; U.S. Patent No. 4,226,848; U.S. Patent No. 4,713,243; U.S. Patent No. 4,940,587; PCT International Application WO 85/02092; European Published Application 205282; Smart JD et al. (1984) *J Pharm Pharmacol* 36:295-9; Sala et al. (1989) *Proceed Intern Symp Control Rel Bioact Mater* 16:420-1; Hunter et al. (1983) *International Journal of Pharmaceutics* 17:59-64; "Bioadhesion - Possibilities and Future Trends, Kellaway," Course No. 470, May 22-24, 1989.

Commercially available sustained release formulations and devices include those marketed by ALZA Corporation, Palo Alto, CA, under tradename ALZET, INFUSET, IVOS, OROS, OSMET, or described in one or more U.S. Patents: No. 5,284,660, issued Feb. 9, 1994; No. 5,141,750, issued Aug. 25, 1992; No. 5,110,597, issued May 5, 1992; No. 4,917,895, issued April 17, 1990; No. 4,837,027, issued June 6, 1989; No. 3,993,073, issued Nov. 23, 1976; No. 3,948,262, issued April 6, 1976; No. 3,944,064, issued March 16, 1976; and No. 3,699,963; International Applications PCT/US93/10077 and PCT/US93/11660; and European Published Applications EP 259013 and EP 354742.

Sustained release compositions and devices are suitable for use in the present invention because they serve to prolong contact between the antigen and the gut-associated lymphoid tissue (GALT) and thus prolong contact between the antigen and the immune system. In addition, sustained release compositions obviate the need for discrete multi-dose administration of the antigen and permit the required amount of antigen to be delivered to GALT in one or two daily doses. This is anticipated to improve patient compliance.

Orally administrable pharmaceutical formulations containing one or more of a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog are prepared and administered to mammals who have manifested symptoms of vascular disorder, such as atherosclerosis. Additionally, subjects who are at risk for developing a vascular disorder, i.e., have a genetic predisposition to developing the disorder,

as determined through suitable means, such as genetic studies and analysis, are treated with similar oral preparations.

Pharmaceutical formulations for oral or enteral administration to treat vascular disorders are prepared from a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog and a pharmaceutically acceptable carrier suitable for oral ingestion. The quantity of a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog in each daily dose may be between 0.01 mg and 1000 mg per day. However, the total dose required for treatment can vary according to the individual and the severity of the condition. This amount can be further refined by well-known methods such as establishing a matrix of dosages and frequencies of administration.

For by-inhalation administration (i.e., delivery to the bronchopulmonary mucosa) suitable sprays and aerosols can be used, for example using a nebulizer such as those described in U.S. Patent Nos. 4,624,251 issued November 25, 1986; 3,703,173 issued November 21, 1972; 3,561,444 issued February 9, 1971; and 4,635,627 issued January 13, 1971. The aerosol material is inhaled by the subject to be treated.

Other systems of aerosol delivery, such as the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman SP in Aerosols and the Lung, SW Clarke SW and D Davis, eds. pp. 197-224, Butterworths, London, England, 1984, can be used when practicing the present invention.

Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources including Fisons Corporation (Bedford, MA), Schering Corp. (Kenilworth, NJ) and American Pharmoseal Co. (Valencia, CA).

Formulations for nasal administration can be administered as a dry powder or in an aqueous solution. Preferred aerosol pharmaceutical formulations may comprise for example, a physiologically acceptable buffered saline solution containing a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog of the present invention.

Dry aerosol in the form of finely divided solid comprising a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog in particle form, which particles are not dissolved or suspended in a liquid are useful in the practice of the present invention. The antigen may be in the form of dusting powders and comprise finely divided particles having an average particle size of between about 1 and 5

μm, preferably between 2 and 3 μm. Finely divided antigen particles may be prepared by pulverization and screen filtration using techniques well known in the art. The particles may be administered by inhaling a predetermined quantity of the finely divided material, which can be in the form of a powder.

Specific non-limiting examples of the carriers and/or diluents that are useful in the pharmaceutical formulations of the present invention include water and physiologically acceptable buffered saline solutions such as phosphate buffered saline solutions pH 7.0-8.0.

The nasally administered formulation of the present invention may include a thermosetting gel which increases in viscosity at body temperature upon contact with the nasal mucosa.

Formulations for buccal administration can include mucoadhesive mixed with effective amounts of a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog. Effective amounts are anticipated to vary according to the formulation employed. For formulation administered by inhalation, the effective amount is likely to be less than that of the oral dose.

Preferably, the duration of treatment in humans should be a minimum of two weeks, and typically three months, and may be continued indefinitely or as long as benefits persist. The treatment may be discontinued if desired (in the judgment of the attending physician) and the patient monitored for signs of relapse. If clinical symptoms or other disorder indicators show that the patient is relapsing, treatment may resume.

As will be understood by those skilled in the art, the dosage will vary with the antigen administered and may vary with the sex, age, and physical condition of the patient as well as with other concurrent treatments being administered. Consequently, adjustment and refinement of the dosages used and the administration schedules will preferably be determined based on these factors and especially on the patient's response to the treatment. Such determinations, however, require no more than routine experimentation, as illustrated in Examples provided below.

Administration of a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog can be conjoined with mucosal administration of one or more enhancers, i.e. substances that enhance the tolerizing effect of the a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog antigen. Such enhancers include lipopolysaccharide (LPS), Lipid A (as described in U.S. Application Serial No. 08/202,677, published as WO 91/01333), IL-4, IL-10 and Type I

interferon (See, e.g., U.S. Application Serial Nos. 08/420,980 and 08/420,979 and WO 95/27499 and WO 95/27500). As used in the preceding sentence, “conjoined with” means before, substantially simultaneously with, or after administration of these antigens. Naturally, administration of the conjoined substance should not precede nor follow administration of the antigen by so long an interval of time that the relevant effects of the substance administered first have worn off. Therefore, enhancers should usually be administered within about 24 hours before or after the a heat shock protein, a therapeutically effective HSP fragment, and/or a therapeutically effective HSP analog antigen and preferably within about one hour.

As used herein the term "therapeutically effective fragment" refers to a peptide or polypeptide containing partial amino acid sequences or moieties of heat shock proteins possessing the ability to treat a vascular disorder. Preferably, such fragments are able to suppress or prevent an inflammatory response upon mucosal administration. Such fragments need not possess the inflammatory properties of the entire heat shock protein. By way of non-limiting example, when MBP is administered parenterally to susceptible mice in the presence of an adjuvant, it induces experimental allergic encephalomyelitis (EAE). It is known that certain non-disease-inducing fragments of MBP (i.e., fragments of MBP which do not induce EAE when administered parenterally with an adjuvant) nevertheless possess autoimmune-suppressive activity when administered orally (or enterally) or in aerosol form to mammals suffering from EAE. Examples of such fragments are reported in U.S. patent application Ser. No. 07/065,734, filed June 24, 1987, and International Patent Application No. PCT/US88/02139, filed June 24, 1988. Therapeutically effective fragments and analogs can be identified by observing a change in cytokine release profile, such as illustrated in the Examples or in other *in vitro* or *in vivo* assays which are predictive of a human vascular disorder and from which agents can be selected which alleviate detectable symptoms of the disorder. Cytokines can be measured using routine assays, including commercially available immunoassays such as radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA).

As employed herein the term "therapeutically effective analogs" of such heat shock proteins or fragments thereof refer to compounds that are structurally related to these heat shock protein peptides or to their therapeutically effective fragments (e.g., inflammatory response-suppressive fragments) and which possess the same biological activity, i.e., the ability to treat the condition, e.g., by eliminating or suppressing the inflammatory response,

upon mucosal administration, either nasally, orally, or enterally. By way of non-limiting example, the term includes peptides having amino acid sequences which differ from the amino acid sequence of the heat shock protein peptide or therapeutically effective fragments thereof by one or more amino acid residues (while still retaining the inflammatory response-suppressive activity of the heat shock protein peptide or fragment) as well as compounds or compositions which mimic the inflammatory response-suppressive activity of the heat shock protein peptide in its ability to suppress or alleviate the symptoms of the disorder.

Fragments and analogs of heat shock protein peptides for use in the present invention can be synthesized using solid phase synthesis techniques well known in the art such as those of Merrifield RB (1962) *Fed Proc Am Soc Exp Biol* 21:412 and *J Am Chem Soc* 85:2149 (1963), and Mitchel AR et al. as well as Tam J et al. (1976) *J Am Chem Soc* 98:7357. Analogs can be constructed by identifying an equivalent amino acid sequence and using the peptide synthesis techniques disclosed above. Therapeutically effective analogs and fragments can also be obtained using recombinant DNA techniques well known in the art.

As used herein the term "inflammatory disease-suppressive agent" is a category of a therapeutically effective agent which refers to a compound or composition which can be administered to a mucosal surface of a mammal to suppress, prevent or delay the clinical onset or manifestation of an inflammatory vascular disease. The term includes heat shock protein peptides that are active against a specific inflammatory vascular disorder, as well as inflammatory disease-suppressive fragments or analogs thereof as defined above.

As used herein the term "vascular disorder" refers to a disease or process involving tissue intrinsic to the blood vessels, particularly the arterial vessels, in which the lumen of affected vessels are narrowed as a result. The archetype of vascular disorder is atherosclerosis. A vascular disorder can involve vessels associated with one or more vascular beds, e.g., the coronary arteries, the cerebral arteries, the aorta, the renal arteries, the splanchnic bed, the peripheral arteries, etc. Included are arterial aneurysms, e.g., aortic aneurysm. Such aneurysms are preferably non-traumatic in origin and can but need not necessarily be atherosclerotic. Also included are a number of principally inflammatory vascular disorders, including but not limited to: allergic angiitis and granulomatosis (Churg-Strauss disease), Behçet's syndrome, Cogan's syndrome, graft-versus-host disease (GvHD), Henoch-Schönlein purpura, Kawaski disease, leukocytoclastic vasculitis, polyarteritis nodosa (PAN), microscopic polyangiitis, polyangiitis overlap syndrome, Takayasu's arteritis, temporal arteritis, transplant rejection, Wegener's granulomatosis, and thromboangiitis

obliterans (Buerger's disease). The measurable symptoms and diagnostic markers of these vascular disorders are well established in the literature and known to physicians practicing in this field. See, for example, Harrison's Principles of Internal Medicine, 14th ed., AS Fauci et al., eds., New York: McGraw-Hill, 1998.

The tolerance induced by the autoimmune-suppressive agents of this invention is dose-dependent. Dose dependency was also seen in the autoimmune arthritis system. Moreover, the mucosal administration of an irrelevant antigen (i.e., one not implicated in an autoimmune disease, such as ovalbumin (OVA) peptide, histone protein, or certain synthetic fragments of MBP) has no effect on the clinical manifestation of the autoimmune disease.

Administration of heat shock proteins via the aerosol route for the treatment of immune disorder has several advantages over other routes of administration. Ease of administration is one important advantage. Also, as shown below in Example 3, aerosol administration of HSP65 is effective in treating atherosclerosis at substantially lower doses than those required to treat this disorder when the same agent was administered orally via the oral route in a solid dosage form. A further advantage is that the aerosol administration route involves less exposure of the heat shock protein peptides of the present invention to degradative gastric juices, which may act to reduce the efficacy of such agents.

It should be noted that the amount of inflammatory response-suppressive agent of the present invention which the treated animal receives via aerosol administration is substantially lower than the total amount of the agent which is administered. It is believed that only 1/200 of the total dosage present in the nebulizer is actually taken up on the pulmonary surface of the treated animals. The majority of the autoimmune suppressive agent nebulized into the cages is not inhaled by the animals but non-specifically adheres to the cages and to the animals. Therefore, aerosol administration is much more effective than oral or enteral administration where in the latter case all of the autoimmune suppressive agent is delivered to the treated animals.

Various animal models have been developed for the study of atherosclerosis and are predictive of human atherosclerosis. Among the more common models are those in which inbred strains of mice have been rendered deficient for either the LDL receptor (LDLR -/-) or apolipoprotein E (apo E -/-) by a gene knockout. The LDL receptor is a 160 kDa glycoprotein responsible for the transfer of LDL out of the plasma and into the cytoplasm of virtually all cell types. The major site of LDL uptake and catabolism is the liver. LDLR -/- mice created on a C57BL/6 background develop accelerated atherosclerosis when fed a high

cholesterol diet, but not when fed a regular chow diet. By contrast, wild-type C57BL/6 mice typically do not develop accelerated atherosclerosis on either a high cholesterol or a regular chow diet.

In one very recent study Afek and coworkers found that LDLR -/- C57BL/6 mice immunized subcutaneously with 10 or 100 μg of heat-killed *Mycobacterium tuberculosis* and maintained on a normal chow diet for three months developed significantly larger fatty streaks than negative control mice immunized with bovine serum albumin. Afek A et al. (2000) *J Autoimmun* 14:115-121. These and other animal models can be used to select HSPs that are useful in accordance with the methods of the invention.

The invention is further described below by reference to examples, the purpose of which is to illustrate the present invention without limiting its scope. All documents cited herein are incorporated by reference.

EXAMPLES

EXPERIMENTAL METHODS

Animals. Wild-type C57BL/6 and LDLR -/- C57BL/6 mice (6 -8 weeks of age) were obtained from the Jackson Laboratory, Bar Harbor, Maine, and used in all experiments.

HSP65. Purified *Mycobacterium bovis* BCG HSP65 was obtained from StressGen Biotechnologies Corporation, Victoria, BC, Canada.

Nasal Administration. The aerosol HSP65 was administered in phosphate buffered saline (PBS, pH 7.4) using a nebulizer. Aerosol was administered to test animals through a hole punched in the side of the cage which held the animals. For aerosolization, a nebulizer (American Pharmoseal Co., Valencia, California, Catalog No. 002038) was attached to an air pressure outlet delivering the equivalent of 7.4 liters of oxygen (the amount of oxygen used in a hospital for nebulization). The nebulizer produced droplets of spray having a diameter of between about 0.3 micrometers and about 0.5 micrometers in diameter. 25 mg of HSP65 was dissolved in 5 ml of PBS. This was then aerosolized over a 10 to 15 minute period to 5 mice per cage (having dimensions 14" x 12" x 7", for height, width and depth, respectively). During aerosolization, a fine mist was created in the cage and the mice moved about freely.

Oral Administration. Heat shock proteins and OVA peptides were orally administered in a 1 ml volume through a syringe fitted with 18G ball-point needle according to the schedules specified in the examples below.

Cell Proliferation Assays. At the specified time, mice were sacrificed and their spleens and popliteal lymph nodes were removed. A single-cell suspension was prepared by pressing the spleens or lymph nodes through a stainless steel mesh. After being washed twice, the cells were resuspended in RPMI 1640 containing 1% glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids, 5% fetal calf serum and $5x10^{-5}$ M 2-mercaptoethanol. The cells were then seeded into a 96-well flat-bottom plate in quadruplicate at a concentration of $2.5x10^{5}$ cells/well and cultured with various concentrations of HSP65 at 37°C with 5% CO_2 for 72 hours. Tritiated thymidine was then added to the culture at 1 μCi/well. The cells were harvested on an automatic cell harvester 6 hours after the pulsing and proliferation was determined by [^{3}H]-thymidine incorporation as measured by liquid scintillation counting.

Cytokine ELISAs. A solid-phase enzyme-linked immunoabsorbent assay (ELISA) was used for determination of concentrations of various cytokines. Microtiter plates were coated with anti-cytokine antibodies (100 ng/well) in 0.1 ml of 0.1M sodium bicarbonate, pH 8.2. Plates were incubated for 18 hrs at 25°C. After 3 washes with PBS/Tween-20 (Bio-Rad), pH 7.5, plates were incubated with 3% BSA/PBS for 2 hrs at 37°C, washed twice, and 100 μl of diluted serum was added in quadruplicate. The plates were incubated for 2 hrs at 37°C. After three rinses with PBS/Tween-20, plates were incubated with 100 μl/well of peroxidase-conjugated goat anti-rat IgG antibody (Tago, U.S.A.) diluted 1:1000 in 1% BSA/PBS for 1 hr at 25°C. Color reaction was obtained by exposure to D-phenylenediamine (0.4 mg/ml phosphate/citrate buffer, pH 5.0) containing 30% hydrogen peroxide. The reaction was stopped by adding 0.4N H_2SO_4 and OD 492 nm was read on an ELISA reader.

Plaque Area Measurements. Quantification of atherosclerotic fatty streak lesions was performed by calculation of lesion size in the aortic arch as previously described. Qiao JH et al. (1994) *Arterioscler Thromb* 14:1480-97. In brief, the heart and upper portion of the aorta were perfused with PBS, removed from the animals, and the peripheral fat was carefully removed. The aortic arch section was embedded in OCT medium and snap frozen. Every other 10 μm thick section throughout the aortic arch was stained with oil red O and taken for analysis. An observer blinded to the treatment counted lesion areas on a calibrated grid. The total area was reported in μm^2.

Immunohistochemistry. Immunohistochemical staining with antibodies to mouse Mac-3 and mouse CD4 (PharMingen, San Diego, California) was performed using 5 μm

cryostat sections of the aortic arch as previously described. Qiao JH et al. (1994) *Arterioscler Thromb* 14:1480-97. Briefly, cryostat sections were air-dried, fixed in acetone, and stained as appropriate with MAC-3 for macrophages, anti-CD4 for T cells, anti-IL-10 (BD PharMingen, San Diego, CA), anti-IFN-γ, or anti-TGF-β (R&D Systems, Minneapolis, MN). Areas that stained positive for macrophages were measured using computer-assisted image quantification (IPLAB Spectrum P 3.1). Lymphocytes identified by anti-CD4 or anti-cytokine antibody staining were counted microscopically by four blinded observers. Results were reported as cells stained/total cells x 100 % as scored above.

Diets. Where indicated, mice were fed a high cholesterol diet (Research Diets; 1.25% cholesterol, 0 % cholate) or a conventional mouse diet (chow).

Statistical Analysis. Data are presented as mean ± SEM. Statistical differences were determined by 1-way ANOVA. $P<0.05$ was accepted as statistically significant.

EXAMPLE 1

Nasal pretreatment with HSP65 leads to a suppressed immune response *in vitro* upon subsequent exposure to HSP65.

Wild-type C57BL/6 mice were treated nasally three times over a week with 0.8 μg HSP65; 8 μg HSP65, or equal amounts of OVA peptide. Three days after the last nasal treatment, mice were immunized subcutaneously with 8 μg HSP65 in conjunction with either CFA (equivalent to 50 μg of *Mycobacterium tuberculosis*) or IFA. All mice were maintained on normal chow diets. Tissues were harvested ten days after immunization for use in the *in vitro* cell proliferation and cytokine assays discussed below.

Cell proliferation assays. Single-cell suspensions from popliteal lymph nodes draining the immunization site were prepared in complete medium and aliquoted into individual wells supplemented with log-fold dilutions of HSP65 to final concentrations ranging from 10 μg/ml to 0.01 μg/ml. Cell proliferation was measured in a ^{3}H-thymidine uptake assay following conventional methods. All measurements were performed in triplicate. Background counts from cells in wells receiving no HSP65 were subtracted from all. Results from this assay are shown in **Figure 1**. As evident from the data presented in **Figure 1**, popliteal cells taken from control mice nasally pretreated with 0.8 μg OVA peptide and immunized with HSP65 in CFA responded vigorously upon challenge with HSP65 at a concentration of 10 μg/ml. In contrast, popliteal cells taken from mice nasally pretreated

with 0.8 μg HSP65 and immunized with HSP65 in CFA exhibited a greatly attenuated proliferative response to HSP65 at a concentration of 10 μg/ml ($p=0.003$ for OVA + CFA versus HSP + CFA). Popliteal cells from mice nasally pretreated with 0.8 μg HSP65 and immunized with HSP65 in IFA proliferated less than half as much as the controls. Proliferative responses to HSP65 introduced at lower concentrations yielded too few counts to make comparisons.

The same assay was also performed using splenocytes, with a similar pattern of findings. Spleen cells taken from control mice nasally pretreated with 0.8 μg OVA peptide and then immunized with HSP65 in CFA responded vigorously upon challenge with HSP65 at a concentration of 10 μg/ml. In contrast, spleen cells taken from mice nasally pretreated with 0.8 μg HSP65 and then immunized with HSP65 in CFA, and likewise spleen cells from mice nasally pretreated with 0.8 μg HSP65 and then immunized with HSP65 in IFA, proliferated about half as much as the controls. Proliferative responses to HSP65 introduced at lower concentrations yielded too few counts to make comparisons.

Interestingly, despite the immunosuppressive effect of nasally pretreating with 0.8 μg HSP65, nasal pretreatment with a ten-fold higher dose of HSP65 (8 μg) appeared to be immunizing. Proliferation data using popliteal lymph node cells in the presence of HSP65 at 10 μg/ml, as above, revealed that cells taken from mice pretreated with 8 μg HSP65 and then immunized with HSP65 in CFA, and cells taken from mice similarly pretreated but then immunized with HSP65 in IFA, both exhibited greater proliferation in response to HSP65 at 10 μg/ml than controls. Thus, nasal pretreatment with the lower dose of HSP65 tended to tolerize, while pretreatment with the higher dose tended to immunize, with respect to HSP65.

Cytokine secretion assays. Single-cell suspensions from popliteal lymph nodes draining the immunization site were prepared in complete medium and aliquoted into individual wells supplemented with HSP65 at a final concentration of 10 μg/ml. After 24 hours of incubation, tissue culture supernatants were prepared by centrifugation and filtration (0.4 μm) for use in ELISAs. Samples were serially diluted in buffer and run in triplicate on ELISA plates with internal standard curve positive and negative controls, following instructions provided by the manufacturer. Static colorimetric measurements were performed using a multiwell plate reader set to the proper wavelength. The different assays measured interferon gamma (IFN-γ), interleukin 6 (IL-6), IL-10, and transforming growth factor beta (TGF-β). Results are shown in **Figure 2**.

Figure 2A depicts the results of IFN-γ ELISA for popliteal lymph node cells taken from mice nasally pretreated with 0.8 μg OVA peptide or HSP65 and subsequently immunized with HSP65 in CFA. Compared to control pretreatment with OVA peptide and subsequent immunization with HSP65 in CFA, IFN-γ was greatly reduced ($p=0.01$) in popliteal cells taken from mice pretreated with 0.8 μg HSP65. In contrast, IFN-γ was increased compared to controls in popliteal cells taken from mice pretreated with 8 μg HSP65 and subsequently immunized with HSP65 in IFA.

IL-6 was likewise reduced in popliteal cells taken from mice pretreated with 0.8 μg HSP65 and subsequently immunized with HSP65 in IFA or CFA, compared to cells taken from control pretreated mice. Nasal pretreatment with 8 μg HSP65 and subsequent immunization with HSP65 in IFA completely suppressed IL-6 secretion in this assay, while similar pretreatment combined with subsequent immunization with HSP65 in CFA had little to no suppressive effect.

Figure 2B depicts the results of IL-10 ELISA for popliteal lymph node cells taken from mice nasally pretreated with 0.8 μg HSP65 and subsequently immunized with HSP65 in CFA. Compared to control pretreatment with OVA peptide and subsequent immunization with HSP65 in CFA, IL-10 was significantly increased ($p=0.04$) in popliteal cells taken from mice pretreated with 0.8 μg HSP65 and subsequently immunized with HSP65 in CFA. IL-10 secretion by popliteal cells from mice pretreated with 8 μg HSP65 was not significantly different from controls.

Secretion of TGF-β by popliteal cells taken from mice nasally pretreated with HSP65 and immunized with HSP65 in IFA was increased compared to controls. Substitution of CFA for IFA led to the opposite result, i.e., TGF-β secretion by these cells was reduced compared to controls.

Similar experiments were also performed using splenocytes. IFN-γ ELISA was performed for spleen cells taken from mice nasally pretreated with 0.8 μg HSP65 or 8 μg HSP65 and subsequently immunized with HSP65 in CFA or HSP65 in IFA. Compared to control pretreatment with OVA peptide and subsequent immunization with HSP65 in CFA, IFN-γ was greatly reduced in spleen cells taken from mice pretreated with 0.8 μg or 8 μg HSP65 and subsequently immunized with HSP65 in IFA. In contrast, IFN-γ was unchanged or increased compared to controls in spleen cells taken from mice pretreated with 0.8 μg or 8 μg HSP65 and subsequently immunized with HSP65 in CFA.

Secretion of TGF-β by spleen cells taken from mice nasally pretreated with HSP65 and immunized with HSP65 in CFA was essentially abolished compared to controls. Substitution of IFA for CFA led to the result that TGF-β secretion by these cells was increased or unchanged compared to controls.

Taken as a whole, data from experiments in this example demonstrate that nasal pretreatment with HSP65 leads to a suppressed immune response upon subsequent exposure to HSP65, with possible contributions from reduced proinflammatory cytokine release and increased anti-inflammatory cytokine release.

EXAMPLE 2

Oral pretreatment with HSP65 leads to a suppressed immune response *in vitro* upon subsequent exposure to HSP65.

Wild-type C57BL/6 mice were treated orally five times over a week with 8 μg HSP65 or equal amounts of OVA peptide. Three days after the last nasal treatment, mice were immunized subcutaneously with 8 μg HSP65 in either CFA (equivalent to 50 μg of *Mycobacterium tuberculosis*) or IFA. All mice were maintained on normal chow diets. Tissues were harvested ten days after immunization for use in the *in vitro* cell proliferation and cytokine assays performed as described in Example 1 and discussed below.

Cell proliferation assay. As shown in **Figure 3**, popliteal lymph node cells from wild-type C57BL/6 mice fed HSP65 (open bars) and subsequently immunized with HSP65 in CFA or IFA proliferated only about half as much as corresponding cells from mice fed OVA peptide (solid bars) and subsequently immunized with HSP65 in CFA or IFA.

Cytokine secretion assays. Experiments measuring IFN-γ, IL-6, and IL-10 were performed, using ELISAs as in Example 1, with popliteal lymph node cells taken from mice fed HSP65 or OVA peptide and subsequently immunized with HSP65 in CFA or IFA. As shown in **Figure 4A**, IFN-γ secretion was strikingly reduced for cells taken from mice fed HSP65 and subsequently immunized with HSP65 in IFA or with HSP65 in CFA. As shown in **Figure 4B**, secretion of IL-6 was also reduced for cells taken from mice fed HSP65 and subsequently immunized with HSP65 in IFA or CFA. IL-10 secretion was minimally affected by feeding of HSP65 in this experimental protocol.

Taken as a whole, data from experiments in this example demonstrate that oral pretreatment with HSP65 leads to a suppressed immune response *in vitro* upon subsequent exposure to HSP65, in association with reduced proinflammatory cytokine release.

EXAMPLE 3

HSP65 treatment reduces atherosclerosis and inflammation in the aortic arch of atherosclerosis-prone LRLR -/- mice treated maintained on a high cholesterol diet.

C57BL/6 mice genetically deficient for LDL receptor (LDLR -/- mice) were divided into three groups: a control group nasally treated with OVA peptide, a group nasally treated with 0.8 μg HSP65 three times over one week, and a group orally treated (fed) 8 μg HSP65 five times over one week. A fourth group had untreated wild-type C57BL/6 mice. Control and HSP65-pretreated mice were then placed and maintained on a high cholesterol diet (HCD) for up to 14 weeks. While on the HCD, nasally pretreated mice were nasally retreated once weekly with 0.8 μg HSP65 or OVA, and orally pretreated mice were refed once weekly with 8 μg HSP65. There was no immunization in this experiment. Aortic arches and spleens were taken at 8, 12, and 14 weeks after initiation of the HCD for immunohistochemical analysis and *in vitro* cell proliferation assays.

Plaque size. Plaque size was significantly reduced in aortic arches of atherosclerosis-prone LDLR -/- mice maintained on a high cholesterol diet following nasal or oral exposure to HSP65. **Figure 5** depicts aortic arch plaque areas (in μm^2) 8 weeks after initiation of HCD. As shown in **Figure 5**, untreated wild-type C57BL/6 mice developed no plaques after 8 weeks on the HCD, while all LDLR -/- mice developed plaques. However, as shown in **Figure 5**, plaques in both the nasally pretreated HSP65 group and the orally pretreated (fed) HSP65 group were significantly smaller than the plaques in the nasally treated OVA group. Plaque size in the nasally pretreated group was smaller than in the orally pretreated group at eight weeks. Plaque size increased in all LDLR -/- mice between 8 and 12 weeks, but the same pattern and significant differences of plaque size were observed.

Aortic arch macrophages. Immunohistochemical staining for macrophages was significantly reduced in aortic arches of atherosclerosis-prone LDLR -/- mice maintained on a high cholesterol diet following nasal or oral exposure to HSP65. **Figure 6** depicts the percent aortic arch area stained with Mac-3 antibodies 8 weeks after initiation of HCD. As shown in **Figure 6**, untreated wild-type C57BL/6 mice had very low level staining, about 1 percent, after 8 weeks on the HCD. All LDLR -/- mice had increased levels of Mac-3 staining at 8

weeks compared to untreated wild-type C57BL/6 mice, with OVA peptide-treated mice having the greatest level at about 12.5 percent. As shown in **Figure 6**, both the nasally pretreated HSP group and the orally pretreated (fed) HSP group had reduced Mac-3 staining compared to the OVA peptide-treated group. Macrophage staining in the nasally pretreated group was about half that in the orally pretreated group, which in turn was about half that in the OVA peptide-treated group at eight weeks. Staining with Mac-3 increased in all LDLR -/- mice between 8 and 12 weeks, but the same pattern and significant differences of staining were observed.

Aortic arch cytokine expression. It is known that in association with the cellular infiltrates, the intima of atherosclerotic plaques express increased amounts of IFN-γ. Aortic arches of atherosclerosis-prone LDLR -/- mice maintained on a high cholesterol diet for 8 weeks following nasal exposure to HSP65 were analyzed by immunohistochemistry for IFN-γ, IL-10, and TGF-β. Scoring by four blinded observers was based on a scale ranging from 0 (no staining) to 3 (maximal staining) for each cytokine. Results are shown in Table 2. Animals treated with HSP65 had less IFN-γ and increased expression of IL-10 as compared to OVA treated mice. TGF-β levels were similar between groups.

Table 2. Cytokine staining in aortic arches of LDL receptor-deficient mice nasally treated with HSP65 and maintained for 8 weeks on high cholesterol diet

	NASAL OVA		NASAL HSP		
Cytokine	No. Positive	Score	No. Positive	Score	
IFN-γ	5/8	1.20	1/8	0.30	P=0.04
IL-10	1/8	1.50	8/8	2.20	P<0.02
TGF-β	4/7	0.60	3/8	0.60	NS

NS, not significant

Aortic arch CD4+ T lymphocytes. CD4+ T lymphocyte staining was significantly reduced in aortic arches of atherosclerosis-prone LDLR -/- mice maintained on a high cholesterol diet following nasal exposure to HSP65. **Figure 7** depicts the percent aortic arch area stained with anti-CD4 antibodies 8 weeks after initiation of HCD. As shown in **Figure**

7, untreated wild-type C57BL/6 mice had very low level staining, about 1 percent, after 8 weeks on the HCD. All LDLR -/- mice had increased levels of CD4 staining at 8 weeks compared to untreated wild-type C57BL/6 mice, with OVA peptide-treated mice having a level at about 12.5 percent. As shown in **Figure 7**, the nasally pretreated HSP group, but not the orally pretreated (fed) HSP group, had reduced CD4 staining compared to the OVA peptide-treated group. CD4 staining in the nasally pretreated HSP group was about half that in the OVA peptide-treated and orally pretreated HSP groups at eight weeks. Staining for CD4 increased in all LDLR -/- mice between 8 and 12 weeks, with the same pattern and significant differences of staining observed.

Spleen cell proliferation assay. Proliferative response to HSP65 was significantly reduced in spleen cells taken from atherosclerosis-prone LDLR -/- mice maintained on a high cholesterol diet following nasal or oral exposure to HSP65. Spleen cell proliferation assays were performed similarly to those described in Example 1, using spleen cells taken from untreated, nasally pretreated, and orally pretreated LDLR -/- mice after 8, 12, and 14 weeks on HCD. The proliferative response to HSP65 was markedly attenuated for splenocytes taken from the nasally and orally pretreated mice at 14 weeks. Proliferation was reduced to very low levels for splenocytes taken from the nasally and orally pretreated mice at 12 weeks. Data for 12 weeks is presented in Table 2.

Cytokine secretion assays. IFN-γ production was significantly reduced (P=0.01) in spleen cells taken from atherosclerosis-prone LDLR -/- mice maintained for 12 weeks on a high cholesterol diet following nasal exposure to HSP65 (25 ± 5 pg/ml), compared to LDLR -/- mice maintained for 12 weeks on a high cholesterol diet following nasal exposure to OVA peptide (170 ± 20 pg/ml). IL-10 was not detectable in spleen cells from either HSP- or OVA-exposed mice. Data is presented in Table 3.

Table 3. Aortic arch staining and in vitro immunologic analysis in LDL receptor-deficient mice nasally treated with HSP65 and maintained on high cholesterol diet for 12 weeks

	NASAL OVA	NASAL HSP	
Aortic Arch Staining			
Plaque Area ($2x\mu m^2$)	8926 ± 4060	4400 ± 2643	P=0.025
Macrophage (% Mac-3+)	20.2 ± 5.9	12.9 ± 6.4	P=0.032
T-Cells (# CD4+ cells)	35.0 ± 7.4	22.3 ± 7.5	P=0.004
IFN-γ (score)	1.30 ± 0.53	1.25 ± 0.52	NS
IL-10 (score)	1.25 ± 0.65	2.20 ± 0.65	P=0.012
TGF-β (score)	0.95 ± 0.82	1.15 ± 0.83	NS
Immune Response (splenocytes)			
Proliferation (Δ cpm)	3671 ± 400	1679 ± 200	P=0.00015
IFN-γ (pg/ml)	170 ± 20	25 ± 5	P=0.01
IL-10 (pg/ml)	ND	ND	NA

NA, not applicable; ND, not detected; NS, not significant

Anti-HSP65 antibody titers and isotypes. LDLR -/- mice maintained for 12 weeks on a high cholesterol diet and mucosally treated with ovalbumin or HSP65 were bled at the end of the experiment, and total IgG, IgG1 and IgG2a were measured by ELISA. Mice nasally treated with HSP65 had significantly lower titers of total IgG and higher values of IgG1 as compared to control treated mice (p=0.05). Mice either nasally or orally treated with HSP65 had decreased titers of IgG2a, however only the orally treated mice had a significant decrease compared to control treated mice (P<0.01). Statistical significance was determined by Student's T test.

Taken as a whole, data from experiments in this example demonstrate that HSP65 treatment reduces atherosclerosis and inflammation in the aortic arch of atherosclerosis-prone LDLR -/- mice treated maintained on a high cholesterol diet.

What we claim is: